Production of biomass and flavonoid of Gynura procumbens (Lour.) Merr shoots culture in temporary immersion system

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Original Article

Production of biomass and flavonoid of *Gynura procumbens* (Lour.) Merr shoots culture in temporary immersion system

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ABSTRACT

Gynura procumbens (Lour.) Merris one of medicinal plant which was carried out used as antioxidant, anticancer, anti-inflammatory, hepatoprotective, and antimicrobial. Many strategies were used to increase the production of biomass and valuable compounds. This study was to investigate the variation effect of growth regulators and immersion frequency on production of biomass and flavonoid contained of *G. procumbens* shoots culture in temporary immersion bioreactor. Stem nodes were used as an explants and induction of shoots were done in solid MS medium supplemented with many kinds of growth regulator. The best treatments were used to produce biomass and flavonoid compounds in temporary immersion bioreactor; there are combination of IAA 2 mg/L and BA 4, 6, 8 mg/L and immersion frequency (5 min each 3 h; 15 min each 12 h). Results showed that the growths of *G. procumbens* shoots in solid MS medium were influenced by supplementation of growth regulators. MS medium supplemented with single cytokinine (6 mg/L kinetin) and combination of auxin (IAA) and cytokinine (BA) caused increasing of shoots growth. Production of biomass of *G. procumbens* in temporary immersion bioreactor was achieved in long immersion interval (12 h) and highest flavonoid production was obtained in combination treatment of immersion frequency 15 min each 12 h and MS medium supplemented with IAA 2 mg/L, BA 8 mg/L.

1. Introduction

Gynura procumbens is one of medicinal plant that has been known to treat many diseases such as, anti-hyperglycemic [1], anti-hypertension [2], antimicrobial, antioxidant, anti-inflammatory, anticancer, cardio protective, and improving fertility [3]. Many kind of secondary metabolites that has been explored from *G. procumbens* are kaempferol, quercetine [4], rutin, myricetin, quercetin, apigenin [5] and stigmaterol [6], they are flavonoid compounds. Many flavonoids in *G. procumbens* were used as phytoalexin that was produced to response of elicitors, so the plant had disease resistant. Many flavonoids have an antioxidant bioactivity.

Secondary metabolites in plant were obtained from roots, stems, leaves, flowers and fruits. Over exploitation of plant to obtain secondary metabolites cause plant in eradication. Besides that, production of secondary metabolite in natural habitat was influenced by plant growth stage, environmental stress, nutrition and plant genetic [7]. Plant tissue culture is an alternative technique to solve these problems because in this system, we controlled nutrition and environmental stress.

In recent years, biomass production of organ cultures has been developed in liquid culture, even to produce secondary metabolite. Micropropagation in liquid culture has been developed in many types of bioreactor such as balloon type bioreactor and temporary immersion bioreactor. Balloon type bubble bioreactor has been successfully done in micropropagation of *Morindacitrifolia* (L.) [8], *Eurycomalongifolia* [9], *Panax ginseng* C.A. Meyer [10,11], *Cyclopiagenistoides* (L.) Vent [12], *Hypericum perforatum* [13], *Aloe barbadensis* [14], and *Dendrobium candidum* Wall ex Lindl. [15]. Plant biomass production in balloon type bubble bioreactor has many profits, such as faster production, good quality, produce higher secondary metabolite and low cost, but in this bioreactor, the organ was submerged, so it will contain more water; this condition called hyperhydricity (a physiological disorder occurring in plant tissue culture characterized by high water retention capacity due to adverse culture condition). Besides that, the culture also became lack of oxygen. Temporary immersion system could solve this problem by way of the immersion frequency. Tissue or organ

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culture will obtain more oxygen and decrease hyperhydricity in the temporary immersion system. There are many researchers have been done, such as production of biomass and secondary metabolite in *Panax ginseng* [16], *Talinumpaniculatum* [17], and *Gynura procumbens* [18]. Successfully of temporary immersion system depend on immersion frequency. Immersion frequency suggested was 5–10 min immersed and 1–12 h frequency [19,20]. Adventitious roots culture of *G. procumbens* in temporary immersion system achieved at immersion frequency 15 min each 12 h [18].

Micropropagation of *G. procumbens* [21] and induced biomass and flavonoid of its plant by sucrose and precursor in shoot and callus cultures have been done [22,23], but shoot culture of its plant in temporary immersion system has not been done. The aims of this research were to know influence of various growth regulators on growth and development of explant in MS solid medium and to know influence of growth regulators and immersion frequency on production of biomass and flavonoid compound in temporary immersion system.

2. Materials and methods

2.1. Plant materials

Gynuraprocumbens (Lours.) Merr was obtained from Botanical Garden Purwodadi, Pasuruan, East Java, Indonesia. Stem nodes were used as an explant which was origin from 3 to 6 before apical shoots.

2.2. Shoots induction in MS solid medium with various growth regulators

Shoot induction in solid MS [24] medium was executed to investigate the best growth regulator which was used in temporary immersion bioreactor. MS solid medium was supplemented with 7 g/L agar, 30 g/L sucrose and pH was adjusted at 5.8 by pH meter (Boeco, Germany). Medium was sterilized by autoclave at 1, 2 atm, 121 °C for 20 min and put in culture bottles with diameter 6 cm. Stems which have 1–2 nodes were sterilized by sodium hypochlorite1% (Bayclin, Johnson, Indonesia) for 5 min and were rinsed by sterile distillated water three times, then cut at each nodes (± 1 cm). Stem nodes were planted in MS solid medium supplemented with various growth regulators; there are single growth regulators: indole acetic acid (IAA), naphtalene acetic acid (NAA), benzyl adenine (BA), kinetin (6-furfuril amino purin) and combination of growth regulators: IAA and BA, NAA and BA, IAA and kinetin, NAA and kinetin. Cultures were maintained at 25 ± 2 °C under continuous illumination 3000 lx (General electric cool white fluorescent tubes) for 28 days.

2.3. Shoots culture in temporary immersion bioreactor

Temporary immersion bioreactor was designed by modification of BIT [25,26]; each bioreactor were filled with 200 mL liquid MS medium supplemented with 30 g/L sucrose and pH was adjusted at 5.8. There are six bioreactors which had combination treatment of immersion frequency (5 min each 3 h; 15 min each 12 h) and combination of growth regulators which was produce high shoot multiplication in solid culture (IAA 2 mg/L and BA 4 mg/L; IAA 2 mg/L and BA 6 mg/L; IAA 2 mg/L and BA 8 mg/L). Six stem nodes which were sterilized by previous method, were planted in each bioreactors. Treatments were replicated three times and cultures were incubated at 25 ± 2 °C under continuous illumination 3000 lx (General electric cool white fluorescent tubes) for 28 days.

2.4. Extraction and identification of flavonoid

Shoots from every treatment were dried at 60 °C for five days and then were grinded. Forty mg of dry shoots were immersed in 10 mL ethanol (Merck) and were heated at 60 °C for 5 min and then were filtered by filter paper. Extracts were concentrated to 2 mL and then were analyzed qualitatively by thin layer chromatography. Ethanol extract (2 mL) of each treatment were concentrated to 1 mL, subsequently the extracts were spotted on silica gel 60 F_{254} (Merck) and eluted using ethylacetate: methanol (4:1). Spots were visualized using UV at 366 nm wavelength.

Total flavonoid content was analyzed by UV colorimetric [5]. Each ethanol extract was taken 900 μL and was added 10 μL aquadest so the final volume of ethanol extract was 1 mL. Sample of each treatment was taken 0.25 mL, and then was added 1.25 mL aquadest and 75 μL of $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and incubated for 5 min. Extract was then added by 0.5 mL 1 M of NaOH and aquadest until volume of solution 25 mL. Absorbance of the mixed solution was measured at 510 nm by UV–Vis spectrophotometer (BOECO S-22, Germany). Cathechin was used as standard compound for the quantification of total flavonoid.

2.5. Statistical analysis

Data of fresh weight, dry weight, shoots length, number of shoots, number of leaves, were analyzed using statistical software program (SPSS 19). Each mean value represented the replicate of three determinations were analyzed using Analysis of Varians one way test ($p < 0.05$). To determine the significant difference between treatments, Duncan test was performed, whereas data from treatments in temporary immersion bioreactor were analyzed using ANOVA two way test ($p < 0.05$) and continue using Duncan test.

3. Results and discussion

3.1. Effect of growth regulator on shoots induction

The explants (stem nodes) had a morphogenetic response to different growth regulators (Table 1). A large amount of leaves were found in plantlets cultivated in medium supplemented with growth regulators in almost all treatments. Shoots multiplication in the medium supplemented with growth regulators were higher than in the medium without growth regulators, except in many treatments such as supplemented with IAA 2 mg/L, NAA 2 mg/L, combination of NAA and BA (2:2 mg/L), combination of NAA and kinetin (2:6 mg/L), combination of NAA and kinetin (2:10).

The highest multiplication of shoot was obtained in medium supplemented with 6 mg/L kinetin, was showed by mean of shoots number per explants (7.0 ± 1.2) and mean of leaves number per explants (27 ± 4.6). Supplementation of kinetin could increase number of shoots and number of leaves higher than BA. Cytokinins are usually known to induce the formation of buds in many in vitro cultured organs. Similar to our research, many researcher showed that cytokinin induced multiple shoot formation [27–30]. The lower and higher concentration of kinetin was decreased the number of shoots and leaves. Low concentrations of kinetin also inhibit induction of adventitious shoot of sweetpotato cv. Brondal [31]. Supplementation of BA could increase number of shoots and leaves; the higher concentrations of BA were inhibiting induction of shoots and number of leaves. It can be seen that the addition of BA as a cytokinin in appropriate concentration is certainly essential for shoot induction and multiplication. Similar to this data was

Table 1
Effect of growth regulator on fresh weight, dry weight, number of shoots, length of shoots, and number of leaves for 28 days culture in solid MS medium.

Growth regulator	Concentration (mg/L)	Fresh weight (g)	Dry weight (g)	Number of shoots	Length of shoots (cm)	Number of leaves
IAA	2	0.02 ± 0.03^{jk}	0.04 ± 0.01^{hj}	1.0 ± 0.0^{a}	3.00 ± 0.42^{ij}	7.3 ± 0.5 ^{cd}
NAA	2	0.12 ± 0.03^{de}	0.02 ± 0.01 ^{cd}	1.0 ± 0.0^{a}	2.40 ± 0.81^{ef}	4.5 ± 0.6^{a}
BA	2	0.30 ± 0.02 ^{lm}	0.05 ± 0.00^{kl}	2.5 ± 0.6^{bc}	2.30 ± 0.29 ^{cd}	13.5 ± 0.6^{gh}
BA	4	0.23 ± 0.01^{jk}	0.04 ± 0.00^{ij}	2.5 ± 0.6^{bc}	2.10 ± 0.06^{ab}	10.0 ± 2.3^{ef}
BA	6	0.16 ± 0.01^{ij}	0.03 ± 0.00^{gh}	3.5 ± 0.6^{cd}	2.10 ± 0.06^{ab}	17.0 ± 1.2^{ij}
BA	8	0.11 ± 0.02^{bc}	0.02 ± 0.00^{bc}	2.0 ± 1.2^{ab}	2.00 ± 0.23^{ab}	11.0 ± 2.3^{ef}
BA	10	0.10 ± 0.01^{ab}	0.02 ± 0.00^{a}	2.0 ± 1.2^{ab}	1.65 ± 0.17^{a}	10.5 ± 1.7^{ef}
Kinetin	2	0.15 ± 0.06 ^{fg}	0.03 ± 0.01 ^{fg}	6.0 ± 0.6^{f}	3.10 ± 0.12^{ij}	19.0 ± 4.6^{kl}
Kinetin	4	0.19 ± 0.05^{ik}	0.04 ± 0.01^{hj}	5.0 ± 1.7^{ef}	2.85 ± 0.06^{ij}	19.5 ± 2.9^{kl}
Kinetin	6	0.19 ± 0.02^{ij}	0.03 ± 0.00^{gh}	7.0 ± 1.2^{ef}	2.70 ± 0.00^{gh}	27.0 ± 4.6^{m}
Kinetin	8	0.16 ± 0.01^{gh}	0.03 ± 0.00 ^{fg}	5.5 ± 0.6^{f}	2.70 ± 0.00^{gh}	22.0 ± 1.2^{m}
Kinetin	10	0.15 ± 0.01 ^{fg}	0.03 ± 0.00 ^{fg}	5.5 ± 0.6^{ef}	2.60 ± 0.00^{gh}	22.5 ± 2.9^{m}
IAA & BA	2:2	0.23 ± 0.05^{jk}	0.04 ± 0.01^{ij}	5.0 ± 1.2^{de}	2.90 ± 0.12^{ij}	18.5 ± 5.2^{kl}
IAA & BA	2:4	0.34 ± 0.12^{m}	0.06 ± 0.02 ^{l}	6.0 ± 1.2^{f}	2.80 ± 0.35^{hi}	25.0 ± 8.1^{m}
IAA & BA	2:6	0.32 ± 0.04^{m}	0.06 ± 0.01 ^{l}	6.0 ± 0.6^{f}	2.95 ± 0.06^{ij}	25.0 ± 4.6^{m}
IAA & BA	2:8	0.31 ± 0.04 ^{lm}	0.06 ± 0.01^{kl}	5.5 ± 0.6^{f}	3.05 ± 0.29^{ij}	25.5 ± 4.0^{m}
IAA & BA	2:10	0.29 ± 0.03 ^{lm}	0.05 ± 0.01^{kl}	5.5 ± 0.6^{f}	2.75 ± 0.29^{gh}	25.5 ± 6.3^{m}
NAA & BA	2:2	0.16 ± 0.01^{ij}	0.03 ± 0.00^{gh}	1.0 ± 0.0^{a}	2.75 ± 0.29^{gh}	9.0 ± 0.0^{ef}
NAA & BA	2:4	0.18 ± 0.02^{ij}	0.03 ± 0.00^{gh}	1.5 ± 0.6^{ab}	2.80 ± 0.00^{hi}	9.5 ± 5.2^{ef}
NAA & BA	2:6	0.19 ± 0.01^{jk}	0.03 ± 0.00^{hj}	2.0 ± 0.0^{ab}	3.00 ± 0.12^{ij}	14.5 ± 0.6^{hi}
NAA & BA	2:8	0.22 ± 0.00^{jk}	0.04 ± 0.00^{hj}	2.5 ± 0.6^{bc}	3.20 ± 0.00^{jk}	19.0 ± 1.2^{kl}
NAA & BA	2:10	0.25 ± 0.03^{kl}	0.05 ± 0.00^{jk}	5.5 ± 2.3^{f}	3.40 ± 0.00^{kl}	21.0 ± 4.6 ^{lm}
IAA & Kinetin	2:2	0.10 ± 0.06^{n}	0.02 ± 0.01^{ab}	1.5 ± 0.6^{ab}	2.40 ± 1.04^{ef}	9.0 ± 4.6^{ef}
IAA & Kinetin	2:4	0.11 ± 0.04^{ab}	0.02 ± 0.01^{bc}	2.5 ± 0.6^{bc}	2.20 ± 0.12^{bc}	9.5 ± 1.7^{ef}
IAA & Kinetin	2:6	0.11 ± 0.05 ^{cd}	0.03 ± 0.01^{de}	2.5 ± 0.6^{bc}	2.35 ± 0.75^{de}	10.0 ± 4.6^{ef}
IAA & Kinetin	2:8	0.13 ± 0.07^{ef}	0.02 ± 0.01^{ef}	3.5 ± 3.5 ^{cd}	2.05 ± 0.64^{ab}	10.5 ± 2.9^{ef}
IAA & Kinetin	2:10	0.18 ± 0.05^{ij}	0.03 ± 0.01^{gh}	3.5 ± 0.6 ^{cd}	2.55 ± 0.17 ^{fg}	12.5 ± 1.7 ^{fg}
NAA & Kinetin	2:2	0.23 ± 0.06^{jk}	0.04 ± 0.01^{ij}	1.5 ± 0.0^{a}	3.95 ± 0.64 ^{l}	8.5 ± 0.6^{de}
NAA & Kinetin	2:4	0.16 ± 0.01^{hj}	0.03 ± 0.00^{gh}	1.5 ± 0.6^{ab}	3.20 ± 0.12^{jk}	9.0 ± 1.2^{ef}
NAA & Kinetin	2:6	0.18 ± 0.01^{ij}	0.04 ± 0.00^{hj}	1.0 ± 0.0^{a}	3.45 ± 0.06^{kl}	9.0 ± 0.0^{ef}
NAA & Kinetin	2:8	0.11 ± 0.02^{bc}	0.02 ± 0.00^{hj}	1.5 ± 0.0^{ab}	3.70 ± 0.35^{kl}	7.5 ± 1.7 ^{cd}
NAA & Kinetin	2:10	0.16 ± 0.01^{ij}	0.03 ± 0.00^{gh}	1.0 ± 0.0^{a}	3.40 ± 0.23^{kl}	5.0 ± 0.0^{ab}
Without growth regulator	0	0.17 ± 0.02^{ij}	0.03 ± 0.00^{gh}	1.0 ± 0.0^{a}	2.95 ± 0.52^{ij}	6.5 ± 0.6^{bc}

showed in *in vitro* shoot regeneration of *Chlorophytum borivilianum* Sant. & Fernandez [32].

Higher multiplication of shoot was also showed in medium supplemented with various concentration of IAA and BA combination. They produce mean of shoots number per explants 5–6 and mean of leaves number per explants 18.5–25.5. This result was higher than other treatments. A higher number of leaves in plant cultivated *in vitro* also showed in the medium containing cytokinin benzyladenine, was observed in lavender [33]. Combination of IAA and BA also induced a higher number of leaves in *Ocimum basilicum* [34].

Combination of NAA and BA influence induction of multiplication of shoots and amount of leaves compare with control (without growth regulator). The highest number of shoots per explants (5.5 ± 2.3) and number of leaves per explants (21.0 ± 4.6) was obtained in medium containing 2 mg/L NAA and 10 mg/L BA combination. The higher the concentration of BA in combination with NAA, the more the number of shoot and leaves produced. This phenomenon also occurs in the addition of combinations of IAA and kinetin. The highest number of shoots per explants (3.5 ± 0.6) and number of leaves per explants (12.5 ± 1.7) was achieved on medium containing 2 mg/L IAA and 10 mg/L kinetin. *In vitro* propagation of *Bambusa arundinacea* (Retz.) Wild, increasing concentration of BA in combination with NAA, and increasing concentration of kinetin in combination with IAA was not followed by increasing number of shoots [35].

Response of stem node explants in medium supplemented with NAA and kinetin combination is not as good as the response to the medium supplemented with IAA and BA combination, and IAA and kinetin combination. Multiplication of shoots and number of leaves was low, but the length of shoot was higher than another treatment and without growth regulator. It's also showed in culture of nodal explant of *B. arundinacea* (Retz.) Wild; induction of shoots was lower than another auxin and cytokinin combination [35]. In this study, supplementation of single auxin (IAA or NAA) could not increased number of shoots and number of leaves, but induce formation large amount of roots (data not shown). Many researcher have been reported roots directly formed from the nodal explants in medium supplemented IBA and NAA combination [36], IBA [37], IBA or IAA [38].

Indicator of growth response was also showed by fresh weight and dry weight; it was showed that the higher fresh and dry weight achieved in medium supplemented with IAA and BA combination; another research also showed high fresh weight in medium supplemented with IBA and BA combination in peppermint micropropagation [39]. Increasing fresh weight also associated with increasing of concentration of calcium in cytosol that was produced by increasing mineral absorption from medium caused by supplemented with BA in high concentration [38]. Supplementation of auxin and cytokinine effectively could influence number of leaves, length of shoots [39–41], and induction of roots faster [42]; even in combination of low concentration of auxin and cytokinine [21].

Medium supplemented with various concentrations of IAA and BA combination has higher multiplication of shoots, length of shoots and number of leaves than other treatments. Therefore various concentrations of IAA and BA combination were used to produce biomass and flavonoid of *G. procumbens* in temporary immersion bioreactor.

3.2. Effect of immersion frequency and growth regulators on shoot induction

Combination treatments of immersion frequency and growth regulator could influence shoot induction of *G. procumbens* in fresh weight, number of shoots, length of shoots, and number of leaves;

we found that the highest number of shoots was achieved in immersion frequency 5 min each 3 h and supplemented by IAA 2 mg/L and BA 4 mg/L; the highest number of leaves was obtained in immersion frequency 15 min each 12 h and supplemented with IAA 2 mg/L and BA 6 mg/L; whereas the highest biomass (fresh weight and dry weight) was obtained in immersion frequency 15 min each 12 h and supplemented with IAA 2 mg/L and BA 4 mg/L (Table 2). Shoots induction of *G. procumbens* in temporary immersion bioreactor could not increase some parameter such as number of shoots, length of shoots, and number of leaves, if these parameters were compared with the same parameters in solid culture (Table 1), but there were significant increasing of fresh weight of shoots, especially in immersion frequency 15 min each 12 h (Table 2).

Combination treatments of immersion frequency 5 min each 3 h and supplemented with IAA 2 mg/L and BA 4 mg/L could induce highest number of shoots, but length of shoots were shortest; whereas the longest shoots were obtained in immersion frequency 15 min each 12 h and supplemented with IAA 2 mg/L and BA 4 mg/L, although it was not a significant different with others treatments (Fig. 1). Similar with this result was shown in propagation of Chinese water chestnut using temporary immersion bioreactor system, the highest multiplication rate was achieved in longer duration of immersion (30 min), but average number of shoots was not significant different with others treatments (immersing the culture every 4, 8, 12, 16, or 24 h for 10 min each) [43].

This study investigated the large-scale propagation of *G. procumbens* using temporary immersion system. The results indicated that shoots growth were higher in immersion frequency15 min each 12 h. In this treatment we found the higher mean of fresh weight, length of shoots, and number of leaves compare with immersion frequency 5 min each 3 h. Although this results not significant different with shoot culture in solid medium, temporary immersion system have many advantage to large-scale propagation because in liquid medium have greater transfer efficiencies [44] and better access to nutrient uptake [12,45,46]. Increasing of biomass of adventitious root culture also showed in*G. Procumbens* and *Talinum paniculatum* in immersion frequency 15 min each 12 h [17]. Shoots regeneration of *Charybdis* sp. were optimal in immersion frequency 5 min each 24 h [47]. Immersion interval 3 h caused explants contact with medium more frequent, so explants were lack of oxygen, although length of immersion only 5 min. This condition also caused explants became hyperhydricity (physiological disorder occurring in plant tissue culture characterized by high water retention capacity) and asphyxia (the extreme condition caused by lack of lack of oxygen), so growth of explants was limited. We found that the long immersion frequency (12 h) have better result than short immersion frequency (3h). The similar result also shown in *in vitro* multiplication of *Eucalyptus globulus*, which was obtained the best multiplication in immersion time 2 min and immersion frequency 12 h [48].

Table 2
Effect of immersion frequency and growth regulator on shoot induction of *Gynuraprocumbens* in temporary immersion bioreactor after 28 days culture.

Immersion frequency	Growth regulators		Fresh weight (g)	Dry weight (g)	Number of shoots	Length of shoots	Number of leaves
5 min each 3 h	IAA: BA	2:4	0.37 ± 0.07^{a}	0.04 ± 0.01^{a}	8.2 ± 2.1^{d}	1.42 ± 0.24^{a}	17.0 ± 2.5^{b}
		2:6	0.38 ± 0.13^{a}	0.04 ± 0.02^{a}	4.4 ± 1.2^{ab}	2.28 ± 0.77^{b}	10.2 ± 4.5^{ab}
		2:8	0.29 ± 0.13^{a}	0.03 ± 0.01^{a}	3.0 ± 1.3^{a}	2.47 ± 0.63^{bc}	9.5 ± 6.3^{a}
15 min each 12 h		2:4	0.71 ± 0.27^{c}	0.05 ± 0.02^{a}	4.6 ± 1.5^{bc}	3.08 ± 0.43^{c}	17.3 ± 7.9^{bc}
		2:6	0.61 ± 0.19^{bc}	0.04 ± 0.01^{a}	6.0 ± 1.7^{c}	2.77 ± 0.31^{c}	21.8 ± 6.7^{c}
		2:8	0.48 ± 0.18^{ab}	0.03 ± 0.01^{a}	4.5 ± 0.5^{bc}	2.90 ± 0.96^{c}	15.0 ± 4.0^{bc}

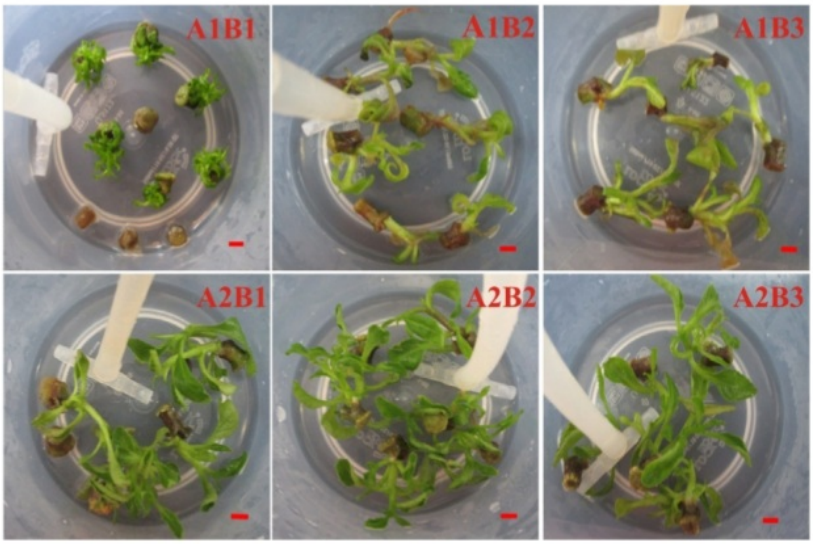

Fig. 1. Effect of immersion frequency and growth regulator on shoot induction of stem explants in temporary immersion bioreactor; A1 = immersion frequency 5 min each 3 h, A2 = immersion frequency 15 min each 12 h, B1 = IAA 2 mg/L and BA 4 mg/L, B2 = IAA 2 mg/L and BA 6 mg/L, B3 = IAA 2 mg/L and BA 8 mg/L; bar = 1 cm.

Table 3
Effect of immersion frequency and growth regulators on flavonoid production.

Immersion frequency	Growth regulators		Flavonoid contained (mg CE/g DW)
5 min each 3 h	IAA:BA	2:4	21.33
		2:6	30.67
		2:8	23.56
15 min each 12 h		2:4	30.67
		2:6	25.33
		2:8	32.00
Ex vitro (mother plant)			5.78

3.3. Effect of immersion frequency and growth regulators on flavonoid production

The highest flavonoid production was obtained in combination treatment immersion frequency 15 min each 12 h and MS medium supplemented with IAA 2 mg/L, BA 8 mg/L. Flavonoid was determined as catechin equivalent (CE) and in this research we found that flavonoid compound in all treatment showed higher than ex vitro shoots (mother plant) (Table 3).

Plants produce a various secondary metabolite compounds that are useful for interacting with the environment and for developing defense systems against stressful conditions and pathogen attacks. Environmental condition such as supplementation of growth regulator and immersed explants in liquid medium can trigger the changes in plant cells that will ultimately result in the accumulation of secondary metabolites that help plants deal with stressful conditions. The stimulus is received by the receptor, which generates secondary messenger activation that transmits signals to the cells through signal transduction pathways leading to gene expression and biochemical changes resulting in secondary metabolite production [49].

Immersion frequency resulted increasing of flavonoid compound in shoot culture of *G. procumbens*, especially in immersion interval 12 h. This is the same condition in adventitious roots culture of *G. procumbens* were treatment by sucrose and various immersion frequency. Long immersion interval (12 h) and immersion duration 15 min in low sucrose concentration provided the highest isoflavon content [18]. Saponin production of *Talinum paniculatum* adventitious roots culture also increased inlong immersion interval [17]. Shoots or adventitious roots could absorb oxygen optimally in glycolysis to produce phosphoenolpyruvate (PEP). The PEP with erythrose 4-phosphate will start shikimic pathway to produce phenylalanine [50].

4. Conclusion

The present study demonstrated that the growths of *G. procumbens* shoots in solid MS medium were influenced by supplementation of growth regulators. MS medium supplemented with single cytokinine (6 mg/L kinetin) and combination of auxin (IAA) and cytokinine (BA) caused increasing of shoots growth. Production of biomass of *G. procumbens* in temporary immersion bioreactor was achieved in long immersion interval (12 h) and highest flavonoid production was obtained in combination treatment immersion frequency of 15 min each 12 h and MS medium supplemented with IAA 2 mg/L, BA 8 mg/L.

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